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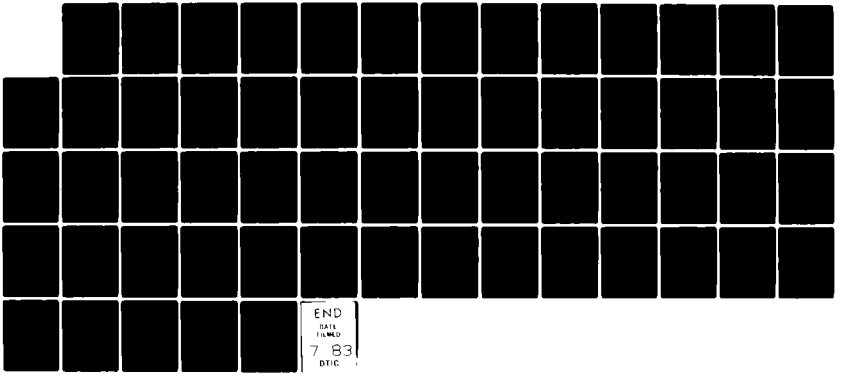

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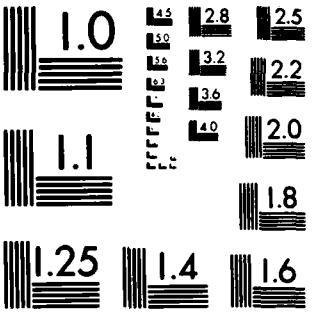

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TRANSITION STATE ANALOG INHIBITORS FOR ESTERASES

(12)

FINAL REPORT

DR. ALAN DAFFORN

JUNE 2, 1983

U.S. ARMY RESEARCH OFFICE

DAAG29-79-C-0048

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acetylcholinesterase; transition state analog inhibitors; mechanism-based inhibitors; anticholinesterases; electrophilic ketones; boronic acids; borinic acids; trifluoromethanesulfonate esters; photoaffinity labelling; acylsilanes

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The effect of electrophilic ketones on acetylcholinesterase activity has been investigated. For example, 1-phenoxy-2-propanone, 1-chloro-3-phenoxy-2-propanone, and 1-fluoro-3-phenoxy-2-propanone are competitive acetylcholinesterase inhibitors with K_I values of 30, 0.85 and 2.2 uM, respectively, compared to 2 mM for 4-phenyl-2-butanone. Substitutent and conformational effects on inhibition suggest that electrophilic ketones bind by formation of a tetrahedral adduct and are transition state analogs.

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20. ABSTRACT CONTINUED

A number of boronic and boronic acids have been found to inhibit acetylcholinesterase with K_I values in the uM range.

Aromatic trifluoromethanesulfonate (triflate) esters have been prepared and determined to be potent irreversible acetylcholinesterase inhibitors. Compounds of this type could be of use as anticholinesterases.

Irradiation of 1-naphthoyltrimethylsilane in the presence of acetylcholinesterase leads to inactivation of the enzyme. Evidence has been developed that this inactivation is active-site-directed and that the acylsilane can be considered a photoaffinity label.

TABLE OF CONTENTS

Statement of Problem Studied

The initial goal of this project was to investigate electrophilic ketones as transition state analog inhibitors of acetylcholinesterase and related esterases. In general, transition state analogs are compounds which resemble the transition state for the reaction catalyzed by the enzyme. Arguments based on absolute rate theory suggest that these compounds should be potent and specific enzyme inhibitors. Since this theory was first seriously advanced about 1970, it has led to the synthesis of powerful reversible inhibitors for a number of enzymes.

More recently, transition state analog theory has become part of a broader concept termed mechanism-based inhibitors; i.e., inhibitors which take advantage of the normal mechanisms of the enzyme to inactivate it. Organophosphate anticholinesterases are a classic example; many of the more recent examples allow the enzyme to generate a strong alkylating agent as an intermediate. This intermediate then alkylates the enzyme and thus inactivates it. As our work has progressed, we have also investigated some of these areas.

Acetylcholinesterase (AChE) was chosen as the target enzyme because of its obvious importance in the nervous system and because it is mechanistically similar in many respects to other members of the serine hydrolases. At the same time, many of the detailed aspects of its mechanisms are still unclear. For example, an unusual rate-determining conformation change has been proposed as the slow step in the acylation reaction under certain circumstances, but the evidence for it is rather indirect. As another example, there is relatively little hard evidence that the basic group in catalysis is a histidine as generally assumed. One overall goal of this project was to prepare mechanism-based anticholinesterases which could be utilized to explore such mechanistic questions.

A second more practical goal was to prepare anticholinesterases of novel structure and intermediate potency as inhibitors. Specifically, transition

state analogs should be much more effective than simple reversible inhibitors such as quaternary ammonium ions, but much less toxic than the more powerful organophosphates. Ideally, they might be expected to be complimentary to the carbamates used pharmacologically and as insecticides.

A third general goal was to explore the mode of binding of these compounds in some detail. In all but a few cases where transition state analogs have been prepared, the actual evidence that they inhibit the enzyme by mimicking some aspect of the transition state is rather scant.

Summary of the Most Important Results

Significant results have been obtained in four areas. Details of this work are described in a publication and in manuscripts in preparation, which are included as Appendices 1-4. The results are summarized below:

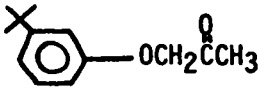
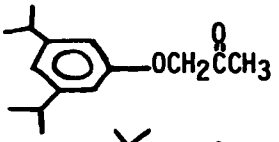
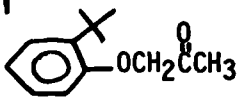
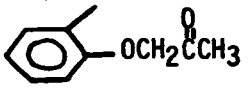
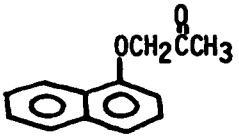
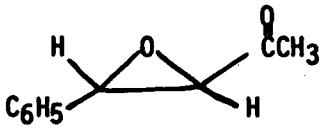
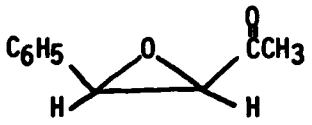
1. Inhibition of AChE by Electrophilic Ketones.

Electrophilic ketones were considered as transition state analog inhibitors because of their ability to form hemiketals. A hemiketal formed by nucleophilic attack of the active site serine hydroxyl of AChE on a ketone may be considered analogous to the tetrahedral adduct formed during ester hydrolysis by the enzyme; thus the ketone should act as a potent reversible inhibitor. In earlier work, the ketone analog of acetylcholine had been prepared and shown to be a good competitive inhibitor, with K_I 10^{-5} M. Present work has been concentrated on uncharged ketones analogous to the substrates phenyl acetate and benzyl acetate and their derivatives. Most of these results are described in the publication included as Appendix 1. Two basic conclusions were reached: first, 1-chloro and 1-fluoro 3-phenoxy-2-propanones are excellent competitve AChE inhibitors with K_I 1 uM; second, several lines of evidence indicate that these ketones bind by hemiketal formation and may be considered transition state analogs.

A number of other ketones have been prepared and examined as inhibitors; the data obtained are presented in Table I. Substitution of a single Cl or Br

Table I

Competitive Inhibition of AChE by Ketones

Compound	K_I (mM)
$C_6H_5CH_2CHBrCCH_3$ (O)	5.0
$C_6H_5CH_2CHClCCH_3$ (O)	0.23
$C_6H_5CH_2CH_2CCH_2Cl$ (O)	0.15
$C_6H_5CHClCCH_3$ (O)	24.0
$C_6H_5CH_2CCH_2Cl$ (O)	3.3
	0.3
	0.15
	0.1
	0.04
	0.1
	0.05
	0.77
$C_6H_5CH_2CH_2CCF_3$ (O)	0.0046
$C_6H_5CH_2CCF_3$ (O)	0.034
$C_6H_5CCF_3$ (O)	0.01

in phenylacetone or 4-phenyl-2-butanone has both an inductive and a steric effect on binding. Introduction of ring substituents in phenoxyacetone generally decreases inhibition compared to phenoxyacetone itself (K_I = 0.03 mM). This result was unexpected, since similar substituents in insecticidal carbamates dramatically increase binding. The difference in substituent effects was shown to result from conformational effects on binding by preparing cis and trans epoxyketones. The trans isomer inhibits about as well as phenoxyacetone, but the cis isomer is a very poor inhibitor. Thus, the enzyme probably binds phenoxyacetone in a trans conformation. Examination of models showed that this conformation is probably different from that adopted by carbamate inhibitors.

Finally, introduction of three or four electron-withdrawing substituents into the phenoxyacetone structure leads to an unexpected decrease in competitive inhibition. For example, 1,1,1-trifluoro-4-phenyl-2-butanone is not as good an inhibitor as 1-fluoro-3-phenoxy-2-propanone (K_I = 4.6 uM vs. 2.2 uM). 1,1,1-trifluoro-3-phenoxy-2-propanone does not appear to inhibit competitively at all. However, this last compound is a powerful irreversible inhibitor with $k_2 = 9 \times 10^3$ M^{-1} min^{-1} (Cf. 3×10^4 M^{-1} min^{-1} for DPF acting on bovine erythrocyte enzyme). The inhibited enzyme can be reactivated by prolonged dialysis. Preliminary experiments suggest that the inhibition can be blocked by 2-PAM, but that 2-PAM will not reactivate inhibited enzyme. All of the results above can be rationalized by assuming that electronegative substituents have two competing effects on ketone binding to AChE. The first effect is stabilization of the hemiketal adduct with the enzyme, leading to stronger binding. However, more extensive electron withdrawal leads to formation of the ketone hydrate in solution. The hydrate cannot form a hemiketal with enzyme and in effect reduces the concentration of inhibitory ketone. In the limiting case of trifluorophenoxyacetone, dehydration of the hydrate becomes rate-limiting. Little competitive inhibition is observed because there is almost no free ketone in solution.

However, the free ketone is an extremely powerful inhibitor; as the ketone is formed from hydrate, it immediately inactivates the enzyme. The overall result is a time-dependent inactivation.

2. Inhibition of AChE by Borinic and Boronic Acids

Borinic and boronic acids have been of interest as transition state analogs for hydrolases because of their ability to form tetrahedral adducts with hydroxyl groups. A few reports have appeared in the literature of relatively weak inhibition of AChE by boronic acids, and the borinic acid analog of acetylcholine has been reported to be a powerful inhibitor. Since borinic acids (R_2BOH) are more closely analogous to the ketones described above and to natural substrates, an investigation of inhibition by these compounds was undertaken for comparison to electrophilic ketones.

Results and discussion of this work are presented in Appendix 2 as a manuscript in prepration. Briefly, a number of these compounds were found to inhibit with K_I in the uM range. No examples were found which inhibit better than the best electrophilic ketones studied earlier. These compounds are of limited use because they are difficult to synthesize and many of them are quite sensitive to air oxidation.

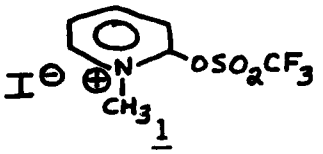
3. Inhibition of AChE by Trifluoromethanesulfonate (Triflate) Esters

This work was prompted by observations in the older literature of AChE inhibition by methanesulfonates. (R. Kitz and I.B. Wilson, J. Biol. Chem. 238, 745 (1963) and references therein). These compounds apparently form a stable methanesulfonate ester of the active serine OH.

Reaction of AChE with corresponding triflate derivatives should produce highly reactive serine triflates in the active site. Further reactions of this triflate should provide information about other nucleophilic groups in the active site, and might allow introduction of new functionalities such as thiols. Accordingly, several triflates were prepared and examined as AChE inhibitors.

Initial work showed that CF_3SO_2Cl is a good irreversible inhibitor, roughly comparable to CH_3SO_2F. However, the kinetics were complex and difficult to reproduce, and the inhibition appeared to be only partially active-site-directed.

Better results were obtained with the quarternary ammonium triflates below:



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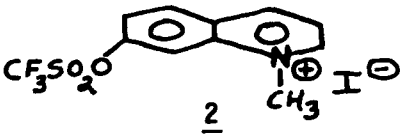

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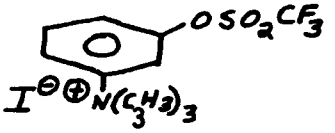

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The synthesis and properties of these compounds are given in Appendix 3. All are irreversible AChE inhibitors with the second order rate constants given in Table II.

Table II

Compound	k_2 (M^{-1} min^{-1})
1	1.4×10^5
2	1.4×10^5
3	1.4×10^2

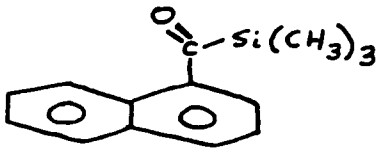
Compounds 1 and 2 are quite potent inhibitors. Compound 1 inhibits AChE about 60 times faster than the methanesulfonate analog and 9 times faster than the carbamate analog pyridostigmine, a medicinally important drug. AChE inhibited by 1 could not be reactivated by phenyl-3-pyridyl antiketoxime methiodide, a compound known to reactivate methanesulfonyl AChE. Thus the initially formed serine triflate in the active site probably undergoes further reaction. No evidence of enzyme activity could be found after treatment of inhibited enzyme with thioacetate; either no thiol was introduced in the active site or the thiol analog of AChE has no activity even toward p-nitrophenyl acetate.

Compounds of this type could be of use as anticholinesterases since they are fairly stable to hydrolysis ($t_{1/2}$ = 8.4 days at pH 7.4 for 2), relatively easy to prepare, and appear to have different structure-activity relationships than

the corresponding carbamates.

4. Photoaffinity Labelling of AChE by a silylketone.

Initial interest in this work was based on the possibility that silylketones might be good AChE inhibitors, since they can be considered electrophilic ketones and they undergo novel rearrangements upon reaction with nucleophiles. Instead, the silylketone 4 was found to inactivate AChE photolytically.



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Considerable evidence has been developed that this inactivation is active-site-directed and that 4 can be considered a photoaffinity label. A manuscript in preparation summarizing this evidence is attached as Appendix 4.

The observed photoinactivation presumably results from generation of a carbene intermediate on photolysis of 4 bound to the enzyme active site. Mechanistic studies of this photolysis suggest that silylketones have several advantages over most of the compounds presently used for photoaffinity labelling and might be generally useful.

LIST OF PUBLICATIONS

Alan Dafforn, John P. Neenan, Charles E. Ash, Laurie Betts, Julie M. Finke, Jeffrey A. Garman, Mohan Rao, Kenneth Walsh, and Robert R. Williams, Bicoehmical and Biophysical Research Communications, 104, 597 (1982).

"Acetylcholinesterase Inhibition by the Ketone Transition State Analogs Phenoxyacetone and 1-Halo-3-Phenoxy-2-Propanones."

SCIENTIFIC PERSONNEL SUPPORTED BY THIS PROJECT AND DEGREES AWARDED DURING THIS REPORTING PERIOD

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APPENDIX I

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ACETYLCHOLINESTERASE INHIBITION BY THE KETONE TRANSITION STATE ANALOGS PHENOXYACETONE AND 1-HALO-3-PHENOXY-2-PROPANONES.

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1-Phenoxy-2-propanone, 1-chloro-3-phenoxy-2-propanone, and 1-fluoro-3-phenoxy-2-propanone are competitive acetylcholinesterase inhibitors with K_I values of 30, 0.85, and 2.2 μM, respectively, compared to 2 mM for 4-phenyl-2-butanone. The substituent effect on inhibition suggests that these compounds bind by formation of a tetrahedral adduct and are transition state analogs.

Other evidence supports this conclusion: N-benzyl-2-chloroacetamide and 1-phenoxy-2-propanol are poor inhibitors (K_I = 11 and >10 mM); 1-phenoxy-2-propanone and 1-chloro-3-phenoxy-2-propanone have K_I values 330 and 140 times smaller than K_m for corresponding substrates; and 1-chloro-3-phenoxy-2-propanone protects the enzyme against irreversible inhibition by CH_3SO_2F.

Transition state analog theory (1) suggests that compounds capable of forming a tetrahedral covalent adduct with the active serine hydroxyl group of acetylcholinesterase should be potent inhibitors, since this adduct should resemble the tetrahedral intermediate formed in ester hydrolysis by the enzyme (2). In earlier work, 4-oxo-N,N,N-trimethylpentanaminium chloride (the ketone analog of acetylcholine) was shown to be a good transition state analog inhibitor (3), presumably by formation of a hemiketal with the enzyme. 4-Phenyl-2-butanone was shown to be a marginal transition state analog (4), and aliphatic ketones were found to be inhibitors but not transition state analogs (5).

The effect of substituents on binding of ketones should provide a useful test for hemiketal formation. Since electron-withdrawing substituents stabilize tetrahedral adducts formed from ketones (6), such substituents should favor hemiketal formation with the enzyme. Substituent effects on binding of

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0006-291X/82/020597-06$01.00/0

4-phenyl-2-butanone derivatives can be compared with effects on benzyl acetate hydrolysis, and are reported here.

Experimental Procedures

General: NMR spectra were recorded on a Varian CFT-20 or A-60. 1-Phenoxy-2-propanone was purchased from Aldrich and 1-phenoxy-2-propanol from Fisher. All other chemicals were of reagent grade. Acetylcholinesterase from Electrophorus Electricus (E.C. 3.1.1.7) was a chromatographically purified preparation obtained from Sigma Chemical Company (activity >1000 units/mg).

Enzyme assays were run on a Radiometer pH Stat system; temperature was maintained at 25° by a Haake FS circulating water bath.

Syntheses: 1-Chlorophenoxypropanone 4 was obtained essentially according to the procedure of Stevenson and Smillie (7). 1-Fluoro-3-phenoxypropanone 6 was prepared by the general route of Bergmann et al. (8); the analytically pure material is a solid with m.p. 49-50°. N-benzyl-2-chloroacetamide (9) was prepared from benzylamine and chloroacetyl chloride. Identity and purity were confirmed by NMR. Structures and compound numbers are given in Table I.

Kinetic Procedures: Competitive inhibition studies were done at pH 7.5 with acetylcholine chloride as substrate in solutions containing 0.1 M NaCl and 0.04 M $MgCl_2$ in glass-distilled water. Methanol was generally added to reaction mixtures to solubilize inhibitors - volume percentages used are given in Table I. Inactivation by methanesulfonyl fluoride was followed by incubating mixtures of enzyme and inhibitors at 25° in 0.1 M NaCl, 0.04 M $MgCl_2$, and 1 mM phosphate buffer, pH 7.0. Aliquots (0.1 ml) were removed and assayed with 1.5 mM acetylcholine chloride. Details of procedures and data treatment have been described previously (4).

Hydrolysis of benzyl chloroacetate by acetylcholinesterase was followed by pH Stat at pH 7.5, 25°, in 0.1 M NaCl, 0.04 M $MgCl_2$, and 5% methanol. Initial velocities were determined over the range 0.2 - 2 mM substrate and kinetic parameters were obtained using a double reciprocal plot.

Hydration of 1-chlorophenoxypropanone 4 was measured in D_2O solution, with the HDO proton decoupled, using a Varian CFT-20 NMR. Chemical shifts assigned by varying D_2O concentration in D_2O/acetone-d_6 mixtures were 4.67 and 5.07 δ (hydrate) and 3.80 and 4.12 δ (ketone) relative to 3-trimethylsilylpropanesulfonate. Integration showed the ketone to be 80% hydrated.

Possible irreversible inhibition by 1-chlorophenoxypropanone 4 was examined by mixing stock solutions of enzyme and inhibitor in a test tube immersed in a 25° water bath. The resulting solution contained 0.1 M NaCl, 0.04 M $MgCl_2$, 1 mM phosphate, pH 7.0, and 25 - 50 units/ml of enzyme. A control contained enzyme and buffer. Aliquots (0.1 ml) were removed from both tubes at various times and added to 25 ml of assay medium (1 mM acetylcholine chloride, 0.1 M NaCl, 0.04 M $MgCl_2$) in the pH Stat. Remaining enzyme activity was measured by the rate of addition of 6 mM NaOH solution at pH 7.0.

Results

Competitive inhibition of acetylcholinesterase by these electrophilic ketones and model compounds is summarized in Table I and shown in Figure 1. Inhibition by 1-phenoxy-2-propanol 3 was barely detectable so only a lower limit for K_I is given. 1-Chlorophenoxypropanone 4 was found to be 80% hydrated

Table I

Reversible Inhibition of Acetylcholinesterase.

Number	Structure	K_I (mM)	%CH_3OH
1	$C_6H_5CH_2CH_2\overset{O}{\overset{\|}{C}}CH_3$*	2	1
2	$C_6H_5OCH_2\overset{O}{\overset{\|}{C}}CH_3$	0.030	1
3	$C_6H_5OCH_2\overset{OH}{\overset{\|}{C}}HCH_3$	>10	2
4	$C_6H_5OCH_2\overset{O}{\overset{\|}{C}}CH_2Cl$	8.5×10^{-4}	0
5	$C_6H_5CH_2NH\overset{O}{\overset{\|}{C}}CH_2Cl$	11	5
6	$C_6H_5OCH_2\overset{O}{\overset{\|}{C}}CH_2F$	2.2×10^{-3}	0

Dissociation constants for competitive inhibition (K_I) were determined in solutions containing 0.1 M NaCl, 0.04 M $MgCl_2$, and the indicated percentages of CH_3OH (v/v) at 25°, pH 7.5. All values are averages of 2 or 3 experiments.
*From Ref. 4

in water by NMR. This ketone is not an irreversible acetylcholinesterase inhibitor even at 1 mM concentration (>1000 times its K_I).

Benzyl chloroacetate was shown to be a substrate for the enzyme, with K_m = 0.11 mM and V_m = 18% of V_m for acetylcholine hydrolysis in 5% methanol.

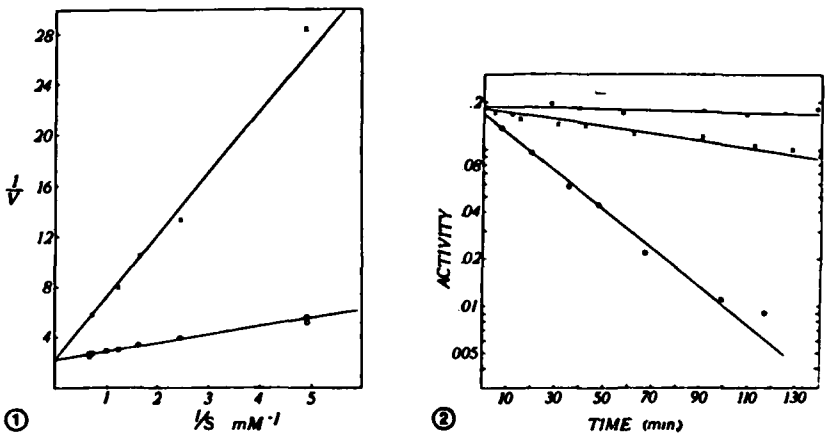

Figure 1. Reversible inhibition of acetylcholinesterase by 1-Chloro-3-phenoxy-2-propanone. Reciprocal velocity vs. reciprocal acetylcholine concentration in the absence (O) and presence (X) of 5.95 μM ketone.

Figure 2. Effect of 1-chloro-3-phenoxy-2-propanone on rate of acetylcholinesterase inactivation by CH_3SO_2F. Remaining enzyme activity is shown as a function of time after mixing for enzyme alone (■), enzyme plus 0.193 mM CH_3SO_2F (O), and enzyme plus 0.193 mM CH_3SO_2F plus 5 μM ketone (X).

Methanol is an extremely weak competitive inhibitor, with K_I = 1.6 M.

1-Chlorophenoxypropanone 4 blocks irreversible inactivation of acetylcholinesterase by CH_3SO_2F, as shown in Figure 2. The dissociation constant for the ketone is 8.2×10^{-7} M, as calculated from its ability to block this irreversible inactivation.

Discussion

The 1-halophenoxypropanones 4 and 6 are potent competitive inhibitors of acetylcholinesterase, although they do not contain the quaternary ammonium group normally introduced to interact with the anionic site of the enzyme. These compounds are roughly comparable as inhibitors to N-methyl-5-hydroxyquinolinium ion and 3-hydroxy-N,N,N-trimethylanilinium ion (10).

The 10^3 enhancement of binding caused by electron-withdrawing substituents (compared to phenylbutanone) is consistent with inductive stabilization of a hemiketal adduct with the enzyme. A quantitatively similar effect is observed in hydration of 1-chlorophenoxypropanone 4. The equilibrium constant for hydration of this ketone is more favorable than that for acetone (6) by a factor of 2.8×10^3, and ketone 4 binds to the enzyme 2.3×10^3 more tightly than phenylbutanone 1. Though data for phenoxyacetone hydration are not available, the intermediate value of its K_I is consistent with the effect of a single electron-withdrawing substituent.

The low K_m observed for hydrolysis of benzyl chloroacetate compared to benzyl acetate (0.11 mM compared to 10 mM) (4) raises the possibility that -Cl may enhance binding to the enzyme by direct hydrophobic or other interactions. However, the similar inhibition by chloro and fluoroketones makes this possibility rather unlikely; these substituents have similar inductive effects but are different in hydrophobicity (11). In addition, the substrate analog N-benzylchloroacetamide 5 inhibits with a K_I comparable to that of N-benzylacetamide-11 and 13 mM, respectively (4). These similarities imply that the -Cl substituent does not contribute to binding, but may lower the K_m for hydrolysis of benzyl chloroacetate (relative to benzyl acetate) by an inductive effect on a kinetic term.

Other criteria developed earlier were used to determine whether these ketones may be considered transition state analogs:

a. A transition state analog should bind more tightly to the enzyme than does the corresponding substrate. The dissociation constant for phenoxyacetone 2 is 330 times smaller than K_m for benzyl acetate hydrolysis. The dissociation constant for 1-chlorophenoxypropanone 4 is 140 times smaller than K_m for hydrolysis of benzyl chloroacetate and 1.3×10^4 smaller than K_I for the substrate analog benzyl chloroacetamide 5. Enhanced binding by factors of 10^2 - 10^4 is typical of transition state analogs (1).

b. Transition state analogs for acetylcholinesterase should block methanesulfonylation of the enzyme. CH_3SO_2F reacts irreversibly with the nucleophilic serine OH group of acetylcholinesterase; compounds which form reversible adducts with this OH group should protect it from CH_3SO_2F. 1-Chlorophenoxypropanone 4 provides this protection (Figure 2); the dissociation constant for the ketone is the same whether calculated from its ability to protect against CH_3SO_2F or from its competitive inhibition of acetylcholine hydrolysis. In earlier work with acetylcholinesterase, those ketones which failed to protect the enzyme against CH_3SO_2F were also not transition state analogs by other criteria (5).

c. Ketone transition state analogs for serine hydrolases should bind more tightly than the corresponding alcohols, since the alcohols cannot form hemiketals with serine. Phenoxyacetone 2 binds more than 330 times more tightly than 1-phenoxy-2-propanol 3.

The observation that 1-chlorophenoxypropanone 4 is not an irreversible inhibitor is somewhat surprising, particularly since it is a good irreversible inhibitor of chymotrypsin (7). This low activity may simply reflect differences in geometry of the active sites.

The observed inductive effects of substituents and the other evidence summarized above suggest one of two plausible mechanisms for inhibition. Either the ketones form tetrahedral adducts with a nucleophile in the active site, or the enzyme binds only the tetrahedral hydrate in a very specific

fashion. The latter interpretation is less likely for several reasons. First, tight binding of the hydrate would suggest that the enzyme operates by direct attack of water on the substrate; but all available evidence indicates instead a nucleophilic attack by the serine -OH group (2). Second, alcohols analogous to these ketones are not tightly bound. Third, highly hydrated trifluoroketones are potent but time-dependent inhibitors of acetylcholinesterase, probably because of the necessity for dehydration before binding (12).[1] Although examination of a broad range of substituents will be required to separate inductive and other effects, the available data are most easily rationalized as a consequence of hemiketal formation between these ketones and the nucleophilic serine hydroxyl group of acetylcholinesterase.

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1. In preliminary work, we have observed very similar time-dependent inhibition by 1,1,1-trifluoro-3-phenoxy-2-propanone.

APPENDIX II

BORINIC AND BORONIC ACIDS AS TRANSITION STATE ANALOG INHIBITORS OF ACTYLCHOLINESTERASE

by

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Introduction

The interaction of boronic acids $RB(OH)_2$ with several serine esterases and proteases including chymotrypsin[1,2,3], subtilisin[4,5,6], and acetylcholinesterase[7,8,9] has been of interest for some time. Boronic acids are generally considered to be transition state analogs for these enzymes.[10] Reaction of the serine-OH in the active site with the electrophilic boron results in an enzyme-boronic acid complex in which the boron has a tetrahedral configuration. This complex resembles the structure of the transition state for ester hydrolysis, in which there is a covalent bond between the oxygen atom of the serine-OH and the original carbonyl carbon atom.[11] Direct support for this suggestion was obtained from an X-ray diffraction study on boronic acid infected crystals of subtilisin.[12] The only known study of the interaction between acetylcholinesterase and a borinic acid (R_2BOH) is reported by Koehler and Hess.[13] They synthesized a boron analog of acetylcholine, the bifunctional borinic acid $(CH_3)_3N^+(CH_2)_3B(OH)CH_3$. This compound is a very potent inhibitor of acetylcholinesterase, with $K_1 = 2.9 \times 10^{-8}$ M (pH 7.5). For comparison with our work on electrophilic carbonyl compounds as possible transition state analogs for acetylcholinesterase[14], it was of interest to consider the boron analogs of ketones and in particular borinic acids. Compared to the corresponding boronic acids, borinic acids might be better inhibitors of acetylcholinesterase as a result of their

more hydrophobic character and closer resemblance to normal substrates. Therefore, we undertook the synthesis of some simple borinic acids, of which few examples are known[15], and examined their activity and that of the corresponding boronic acids as acetylcholinesterase inhibitors.

Materials and Methods

Synthesis

General: Because of the potential toxicity of the desired compounds, all aqueous and waste organic layers were treated with an alkaline solution of 10% H_2O_2 before disposal (except for the acidic layers where only 10% H_2O_2 was used). Glassware was rinsed with the same solution before cleaning.

THF* was dried over and distilled from KOH pellets, then stored over sodium. Dichloromethane was distilled from P_2O_5 and stored over molecular sieves 4 A°. Trimethyl borate and n-propanol were reagent grade and used without further purification.

NMR spectra** were recorded with $CDCl_3$ as solvent and TMS as internal standard (unless otherwise noted). ^{1}HNMR spectra were obtained on a Varian EM-360L spectrometer, and ^{13}C NMR spectra on a Varian CFT-20 spectrometer (reference line 1538 Hz). Mass spectra were determined with a Varian MAT CH7 mass spectrometer at an ionization potential of 70 eV.

General Procedure: In an N_2-atmosphere, a THF solution of the Grignard was added to a stirred THF solution of trimethyl borate, $B(OMe)_3$ or the necessary boronate ester, $Ph(CH_2)_nB(OPr)_2$, cooled to -70°C. The mixture was stirred overnight without further cooling. During the addition, a precipitate generally formed which, when preparing a borinic ester, had dissolved when the temperature of the mixture reached room temperature. Crushed ice was then added, followed by sufficient ice-cooled concentrated HCl to give a strongly acidic reaction to wet pH paper. Extraction with 4 - 5 small (100 ml) portions of ether and washing of the combined organic layers with two 50 ml portions of a saturated NH_4Cl solution kept most of the THF in the aqueous phase. For optimal use of the stabilizing effect of water against oxidation, the organic layer was dried quickly (10 min.) over $MgSO_4$ shortly before conversion into the propyl ester.

The ether was removed from the filtered solution by distillation at aspirator pressure (receiver cooled at -20° to -30°). Then an excess of about 2 molequivalents (calculated on starting material) of n-propanol was added to the residue. Water was removed through azeotropic distillation with CH_2Cl_2 in a Soxhlet apparatus containing activated molecular sieves 4 A° in the extraction thimble. After 5 "extractions" the CH_2Cl_2 solution was concentrated at aspirator pressure and the residue quickly distilled under reduced pressure as a

first purification step. Careful distillation of the collected low boiling fraction through a short column gave the desired compound. The purity was checked by ^{1}HNMR and whenever possible (autoxidation) by the TLC analysis of a solution of the ester in ether saturated with water.

No extensive attempts were undertaken to obtain optimal yields of the desired compounds.

Phenylbis(n-propoxy)borane[17] (1A) Previously distilled bromobenzene, 47 g, (0.3 mole) in 100 ml THF was reacted with 24 g (1.0 mole) of magnesium. After the reaction started (added iodine crystal also as indicator) more THF was added to give a total volume of around 200 ml. The temperature of the mixture was maintained between 20° and 30°C. When the addition of the bromide was completed (5 h) the magnesium was allowed to settle, and the dark colored Grignard solution was filtered through glass wool into a dropping funnel. Reaction with 50 g (0.5 mole, 50 ml) $B[OMe]_3$ in 150 ml THF, as described in the general procedure gave after the second distillation, 45 g (0.22 mole, 70%) of propyl benzeneboronic ester 1a: bp 59° -61°C (2 Torr) (lit. 112 - 113°/10 Torr, Ref. 18). ^{1}HNMR: δ, 0.92 (t, J = 7.0 Hz, 6H); 1.2 - 1.9 (m, 4H), 3.93 (t, J = 6.5 Hz, 4H); 7.1 - 7.9 (m, 5H). ^{13}C NMR[19] 10.00 ($-\underline{CH_3}$); 24.8 ($-\underline{CH_2}-$); 65.8 ($O-\underline{CH_2}-$); 127.8, 129.1, 133.2. Mass spectrum* m/e; 206 (4)* 205 (1) int 100% (M^+), 147 (4) 146 (1) int 28% (M^+ -59[OPr])

Benzylbis(n-propoxy)borane 1b) Benzylmagnesium chloride was prepared from 38 g (0.3 mole) of previously distilled benzyl chloride dissolved in 100 ml THF and 24 g (1.0 mole) of magnesium. After the Grignard reaction started, the total volume of the reaction mixture was raised to about 200 ml of THF. The temperature of the reaction was maintained just below 30° during the addition of the chloride. After the addition, stirring was continued for 5 hrs. When the magnesium settled down, the dark solution was filtered through glass wool into a dropping funnel. Hydrolysis of a small volume, and extraction of the organic products, which were identified by ^{1}HNMR, indicated that no more than 10 mol percent of dibenzyl was present compared to benzylmagnesium chloride (analyzed as toluene). Reaction of the Grignard reagent and 50 g (0.5 mole, 50 ml) $B(OMe)_3$ in 150 ml THF as described in the general procedure, followed by two distillations, yielded 22 g (0.1 mole, 40% calc. on Grignard) propylbenzyl boronic ester: bp 75 - 78°C (2 Torr), contaminated with 10 mol percent dibenzyl. ^{1}HNMR: δ: 0.87 (t, J = 7.0 Hz, 6H); 1.2 - 1.8 (m, 4H); 2.27 (s, broad, 2H); 3.80 (t, J = 6.5 Hz, 4H); 7.20 (s, > 5H), singlet at 2.90 δ characteristic for dibenzyl. ^{13}C NMR δ: 10.3 ($-\underline{CH_3}$), 24.9 ($\underline{CH_2}-$), 65.3 (OCH_2-), 124.7, 128.2, 129.2, 139.8 (C-1). Mass spectrum m/e: 220 (4) 219 (1) int. 100% (M^+), 129 (4) 128 (1) int. 190% (M^+-91)[$PhCH_2$]).

(2-Phenyl)ethylbis(n-propoxy)borane (1c) The Grignard reagent was prepared from 42 g (0.23 mole) of previously distilled 2-phenylethyl bromide, as for benzyl magnesium chloride. Hydrolysis of a small sample of the filtered Grignard-containing solution showed almost no dimeric product present (2%). The Grignard was reacted with 42 g (0.4 mole) $B(OMe)_3$ as described in the general procedure. The second distillation gave 31 g (0.13 mole, 56%) of propyl 2-phenylethylboronic ester; bp 85 - 86°C (2 Torr). ^{1}HNMR δ: 0.6 - 2.0 (m, 12H); 2.75 (t, J = 8.0 Hz, 2H); 3.75 (t, J = 6.5 Hz, 4H); 7.25 (s, 5H). ^{13}C NMR δ: 10.4 ($-\underline{CH_3}$); 25.0 ($OCH_2\underline{CH_2}-$); 30.6 ($Ph-\underline{CH_2}-$); 65.1 ($O\underline{CH_2}-$); 125.5, 128.1, 128.3, 145.4 (C-1). Mass spectrum m/e: 234 (4) 233 (1) int. 100% (M^+), 129 (4) 128 (1) int. 100% (M^+-105 [$PhCH_2CH_2$]).

Methylphenyl(n-propoxy)borane (2a) The n-propyl ester of benzeneboronic acid (21 g, 0.1 mole) (1a) was dissolved in 100 ml THF. Then, 100 ml of a THF solution of CH_3MgBr (1.05 M) was added through a dropping funnel. The reaction and work up followed the general procedure. Care should be taken to avoid high temperatures during concentrating of solutions containing the desired product because of its low boiling point. Second distillation, during which the receiver was cooled in an acetone/CO_2 bath, gave 10.5 g (0.065 mole, 65%) of the propyl ester of phenyl methyl borinic acid 2a: bp 35 - 37°C (2 Torr) (lit. 87 - 89°C; 9 Torr, Ref. 21). ^{1}HNMR δ: 0.73 (s) + 0.98

(t, J = 7.0 Hz) together 6H, 1.3 - 2.1 (m, 2H); 3.98 (t, J = 6.5 Hz, 2H); 7.1 - 7.5 (m, 3H); 7.6 - 8.0 (m, 2H). ^{13}C NMR δ: 10.4 (-$\underline{CH_3}$); 25.1 (-$\underline{CH_2}$-); 67.9 ($\underline{OCH_2}$-); 127.6, 136.8, 134.1. Mass spectrum m/e: 162 (4) 161 (1) int. 100% (M^+); 147 (4) 146 (1) int. 100% (M^+-15 [CH_3]).

(Isopropyl)phenyl(n-propoxy)borane (3a): The propyl ester of benzeneboronic acid (1a), 21 g, 0.1 mole, was reacted as described in the general procedure with 90 ml of a 1.2 M isopropylmagnesium bromide solution in THF (0.108 mole). The second distillation gave 12 g (0.064 mole, 64%) of the propyl ester of phenyl isopropyl borinic acid 3a, bp 46 - 48°C (2 Torr). ^{1}HNMR δ: 0.8 - 2.0 (m, 12H); 3.97 (t, J = 6.5 Hz, 2H); 7.1 - 7.8 (m, 5H). ^{13}C NMR δ: 10.3 ($CH_2\underline{CH_3}$); 18.4 ($CH\underline{CH_3}$); 25.3 (-$CH_2\underline{CH_2}$-); 66.2 [B-$\underline{CH}$](CH_3)$_2$; 68.4 ($\underline{OCH_2}$-); 127.6, 129.1, 132.6, 133.5 (C-1). Mass spectrum m/e: 190 (4) 189 (1) int. 100% (M^+); 147 (4) 146 (1) int. 700% (M^+ -43 [i-Pr]).

(o-Methoxybenzyl)bis(n-propoxy)borane (5b) o-Methoxybenzyl chloride, 28 g, 0.18 mole, (prepared via $LiAlH_4$ reduction of o-methoxybenzoic acid and subsequent $SOCl_2$ treatment of the product, bp 58°C/2 Torr) in 100 ml THF was reacted with 12 g (0.5 mole) of magnesium. After the reaction started, the volume of the reaction mixture was raised to 400 ml, and the reaction temperature maintained at around 5°C.

The greyish Grignard was filtered and reacted with 50 g (0.5 mole) $B(OMe)_3$ in 150 ml THF according to the general procedure. Distillation yielded 18.5 g (0.074 mole, 41%) of propyl (o-methoxybenzyl) boronic ester 5b: bp 104 - 107°C (2 - 3 Torr). ^{1}HNMR δ: 0.85 (t, J = 7.0 Hz, 6H); 1.1 - 1.9 (m, 4H); 2.18 (s, broad, 2H); 3.6 - 4.0 (t + s, 7H); 6.6 - 7.4 (m, 4H). ^{13}C NMR δ: 10.3 ($-CH_2\underline{CH_3}$); 55.2 (OCH_3); 65.3 ($O\underline{CH_2}-$); 110.1, 120.6, 126.0, 129.3, 130.8, 157.4. Mass spectrum m/e: 250 (4) 249 (1) int. 100% (M^+); 191 (4) 190 (1) int. 50% (M^+ -59 [PrO]).

2-(o-Methoxyphenyl)ethylbis(n-propoxy)borane (5c):

2-(o-Methoxyphenyl)ethyl chloride, 23 g, 0.13 mole, (prepared by $LiAlH_4$ reduction of o-methoxyphenylacetic acid and subsequent $SOCl_2$ treatment of the product: bp 90°C/2 Torr) dissolved in 100 ml THF was reacted with 12 g (0.5 mole) magnesium in a total reaction volume of 300 ml THF. It was necessary to keep the reaction temperature around 60°C. The dark colored Grignard solution was reacted with 50 g (0.5 mole) of $B(OMe)_3$ in 150 ml THF according to the general reaction procedure. Distillation gave 22 g (0.08 mole, 60%) of propyl o-methoxyphenylethyl boronic ester 5c; bp 110 - 112°C (2 Torr). ^{1}HNMRδ: 0.7 - 1.9 (m, 15H); 2.5 - 2.9 (m, 2H); 3.75 (t, J = 6.5 Hz) + 3.80 (s) together 7H, 6.8 - 7.4 (m, 4H). ^{13}C NMR δ: 10.0 ($-CH_2\underline{CH_3}$); 24.6 ($-\underline{CH_2}CH_3$); 54.7 ($O\underline{CH_3}$); 64.6

($\underline{OCH_2}$-); 109.8, 120.1, 126.3, 129.0, 133.3, 157.2. Mass spectrum m/e: 264 (4) 263 (1) int. 100% (M^+); 204 (4) 203 (1) int. 500% (M^+ -74 [CH_3 + OPr]).

Methyl[2-(o-methoxyphenyl)ethyl](n-propoxy)borane (6c)

The propyl ester of o-methoxyphenylethyl boronic acid (11.6 g, 0.05 mole) in 100 ml THF was treated with 45 ml of a 1.2 molar CH_3MgBr solution in THF (0.054 mole). The reaction and work up was according to the general procedure. Distillation gave 5.0 g (0.023 mole, 46%) of the propyl ester of methyl o-methoxyphenylethyl borinic acid: bp 80 - 81°C (2 Torr). ^{1}HNMR δ: 0.38 (s, 3H); 0.7 - 1.9 (m, 7H); 2.71 (t, J = 7.0 Hz, 2H); 3.6 - 4.0 (t + s, 5H); 6.6 - 7.3 (m, 4H). ^{13}C NMR δ: 10.3 (-$\underline{CH_2}CH_3$); 24.9 (-$\underline{CH_2}CH_3$); 55.1 ($\underline{OCH_3}$); 67.3 ($\underline{OCH_2}CH_2$-); 110.3, 120.5, 126.5, 129.2, 133.8, 157.5. Mass spectrum m/e: 220 (4), 219 (1), int. 100% (M^+); 205 (4), 204 (1), int. 170% (M^+ -15 [CH_3]; 145 (4), 144 (1) int. 160% (M^+ -75 [CH_3 + HOPr]).

Kinetic Procedure

Acetylcholinesterase (E.C. 3.1.1.7) from electric eel was a chromatographically purified material obtained from Worthington Chemical Co. Inhibition by boronic and borinic esters of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine chloride was measured with a Radiometer pH Stat, using 6 mM NaOH solution as titrant. All experiments were

done in a nitrogen atmosphere at 25°C and pH 7.5. The 30 ml reaction mixture contained 0.04 M $MgCl_2$ and 0.1 M NaCl in glass-distilled water. The concentration of acetylcholine chloride ranged from 0.18 - 1.0 mM, with experiments carried out at five to eight different concentrations. The concentration of enzyme in the reaction mixture was 0.4 - 2.0 x 10^{-12} M, and the hydrolysis reaction was initiated by adding 0.1 ml of an enzyme stock solution to the reaction mixture.

The activity of each inhibitor was studied in at least two separate experiments, before which the compound was carefully distilled. In no case was a difference found between the K_1 values obtained for each compound within experimental error. Stock solutions of the compounds in 0.04 M $MgCl_2$ and 0.1 M NaCl were made up with MeOH at such a concentration that there was less than 0.1% (v/v) MeOH present during the kinetic experiment. Because of the oxidative decomposition of phenylisopropylborinic acid 3a into less active compounds such as benzeneboronic acid 1a, this borinic acid was handled as a 0.15 M solution in methanol, kept in a septum-sealed flask. The inhibitor 3a was introduced via a microsyringe (2 - 6 µl) into the assay mixture containing salts and acetylcholine chloride at pH 7.5. The enzyme was added when the pH was again at 7.5 (after about 0.5 - 1 min.). In order to exclude any change in activity of the enzyme as well as of the inhibitors, duplicate points were determined for the blank and for the curves with different inhibitor concentrations at the end

of the experiment. The difference in observed activity was in all cases within the error limit of the assays. Possible change in inhibitory potency was also studied by varying the time before adding the enzyme to the reaction mixture from 1 min. to 1 hr. after addition of inhibitor. No change in activity of the inhibitors were detected, except for 3a (see above) and very slow decomposition of benzylboronic acid 1b. Time-dependent inhibitory properties were only observed with the o-methoxyphenyl substituted compounds 5b, 5c, and 6c. Inhibition increased with time for a short period after addition of the enzyme to the reaction mixtures containing substrate and inhibitor, as evidenced by unusual curvature in the recording of titrant versus time. Dissociation of these enzyme-inhibitor complexes also required an observable short period since enzyme incubated with inhibitor and diluted into a large volume of substrate gave assays showing increasing activity with time. Both effects appear to have observed half-lives of the order of 20 - 30 seconds under these conditions; detailed kinetic analysis did not give reproducible results because of short-term fluctuations after mixing and the small size of the effect. Steady state kinetics for the o-methoxyphenyl substituted compounds 5b, 5c, and 6c could be obtained as with the other compounds by using the rate of hydrolysis 2 minutes after the addition of the enzyme. Recalculation of the acetylcholine chloride concentrations, especially in the low concentration range, was necessary because of the amount hydrolyzed during this period.

Benzeneboronic ester 1a inhibits only at concentrations higher than 5 mM. Since this inhibition is time-dependent, it may result from the known irreversible inhibition by n-propanol, which is released on hydrolysis,[22] and was not examined further. The boronic acids 1b and 1c, having no substituent in the aromatic ring, are purely competitive inhibitors. The other five boron compounds, boronic acids 5b and 5c and borinic acids 2a, 3a, and 6c, appear to be best described as mixed type inhibitors of enzymatic acetylcholine hydrolysis. The straight lines in double reciprocal plots intersect in one point slightly left of the vertical axis (see Fig. 1 for compound 6c). The inhibition constant for the competitive component K_c was calculated from the slope of the lines in the double reciprocal plot according to the formula:[23]

$$\text{slope} = \frac{K_m}{V}\left[1 + \frac{(I)}{K_c}\right]$$

Replots of slope versus (I) gave straight lines and comparable values for K_c. This graphical method for determining K_c was used with the o-methoxyphenyl substituted compounds 5b, 5c, and 6c because inhibition experiments with these compounds were done for at least 4 different inhibitor concentrations. Because of the small noncompetitive component in the inhibition (< 10%), only the ratio K_c/K_n was determined from the

double reciprocal plot, utilizing the projection of the intersection point of the lines on the horizontal axis ($-K_c/[K_n \times K_m]$).[23] Re-analysis of the data for compounds 2a and 6c by the non-parametric method of Cornish-Bowden and Eisenthal[24] confirmed the presence of a small noncompetitive component.

The ability of one of these compounds, borinic acid 2a, to block the time-dependent irreversible inactivation of the enzyme by methanesulfonyl fluoride[25] was also examined. Reaction conditions are given in Figure 2. Details of the procedure have been discussed previously.[26]

The solutions containing the boronic acid 2a and CH_3SO_2F showed slower loss in activity compared to the solution containing only enzyme and CH_3SO_2F. The loss of activity in both runs followed pseudo-first order kinetics (Fig. 2). The bimolecular rate constant for inactivation of the enzyme by CH_3SO_2F, $k = 2.25\ M^{-1}\ s^{-1}$ is comparable to the value of 2.5 $M^{-1}\ s^{-1}$ found by Kitz and Wilson[25] under somewhat different conditions. The loss of enzyme activity in the blank was negligible.

From the psuedo-first order rate k_1 for solutions containing 2a, an equilibrium constant K_d for the equilibrium between enzyme and borinic acid 2a can be calculated with the formula:[26] $K_d = [I]/[k_o/k_i)-1]$, where k_o is the rate constant in the absence of inhibitor. This analysis gives $k_d = 2.45 \times 10^{-5}$ M at [2a] = 0.049 mM and $K_d = 2.3 \times 10^{-5}$ M at [2a] = 0.095 mM.

Results

Synthesis:

The boronic and borinic acids were prepared according to procedures known in the literature[15], by reaction of a Grignard reagent with trimethylborate or the necessary boronate ester 1.

$$Ph(CH_2)_nMgBr + B(OMe)_3 \xrightarrow[THF,\ -70°]{} \xrightarrow[2.\ PrOH]{1.\ H_3^+O} Ph(CH_2)_nB(OPr)_2$$

1a n = 0 1

1b n = 1

1c n = 2

$$Ph(CH_2)nB(OPr)_2 + RMgBr \xrightarrow[THF,\ -70°]{} \xrightarrow[2.\ PrOH]{1.\ H_3^+O} Ph(CH_2)_n\underset{OPr}{BR}$$

2a n = 0

2b n = 1 R = CH_3 3a n = 0 R = $CH(CH_3)_2$

2c n = 2

Boronic and borinic acids are crystalline compounds but more sensitive toward air oxidation than the corresponding esters.[27] The esters of the boronic and borinic acids have

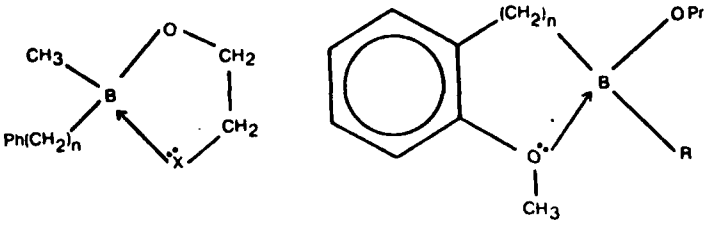
relatively low boiling points; this property makes purification of the esters through distillation very attractive. Therefore, the desired boronic and borinic acids were isolated as their corresponding propyl esters 1, 2, and 3.*

Of the boronic esters 1 , the most difficult to obtain pure was the benzylboronic ester 1b. The propyl ester of benzyl boronic acid was always contaminated with small amounts (up to 10 mol%) of dibenzyl, despite precautions, such as a relatively low reaction temperature (30°C) and high dilution. Purification via careful distillation was unsuccessful, while the recovery of the boronic acid from an alkaline aqueous solution was very low, probably due to autoxidation of the acid.[29] The isolation of pure boronic esters 1a and 1c was straightforward.

The major difficulty encountered in the synthesis of the borinic esters was oxidation or disproportionation. Of the boronic esters 2, it was only possible to obtain the known[21] propyl ester of phenyl methyl borinic acid 2a as a pure compound. The benzyl methyl 2b and phenylethyl methyl ester 2c are formed in the reaction of the Grignard reagent with the boronic ester, but purification through distillation did not result in the isolation of the pure compounds. Studies on these impure fractions showed that these borinic acids undergo rapid oxidation even in aqueous solution (water stabilizes against autoxidation).[29] ^{1}HNMR spectra of the organic extracts from a stirred suspension of these impure fractions

in water showed the disappearance with time of the CH_3B resonance signal around 0.3 δ. The decomposition of the borinic acid was also deduced from the observation that the characteristic blue color[30,31] of an aqueous solution of impure borinic acid 2b or 2c and diphenylcarbazone (from a dilute, slightly orange stock solution of the carbazone in methanol) slowly faded away. The ability of aqueous solutions of these impure borinic esters to inhibit acetylcholinesterase changed rapidly with time. No further attempts were made to purify these compounds.*

Because of our interest in borinic acids, several approaches were taken to decrease their oxygen sensitivity. Introducing steric hindrance around boron[27,32] by substituting the methyl group with an isopropyl group did not give the desired result. Only the phenyl isopropyl borinic ester 3a could be obtained as a pure compound. In agreement with the reported greater oxygen sensitivity of the bond between boron and a secondary carbon atom compared to the bond between boron and a primary carbon atom[27], an aqueous solution[15,29] of the borinic ester 3a lost its inhibitory activity towards acetylcholinesterase fairly rapidly (within half an hour in a typical experiment). This observation is in sharp contrast with the observed remaining activity of an aqueous solution of the borinic ester 2a even after one month storage without any special precautions. ^{1}HNMR experiments and tests with diphenylcarbazone as described for 2b and 2a confirmed the disappearance of the borinic acid 3a in aqueous solution.



4

5b	n = 1	R = OPr
5c	n = 2	R = OPr
6b	n = 1	R = CH_3
6c	n = 2	R = CH_3

Stabilization of the borinic acids via intermolecular coordination was studied in solution by addition of N-methylimidazole. However, the coordinated borinic acid 3a appeared to be a weaker inhibitor by about a factor of 10. Intramolecular coordination was also considered, as in structure 4, since the known ethanolamino esters (X = NH_2) of borinic acids are very stable compounds and can be stored almost indefinitely without special precautions.[33,34] Preparation of the enthanolamino esters of the borinic acids 2b and 2c from the impure propyl esters was unsuccessful. Esters containing more strongly coordinating ligands, such as 8-quinolinol esters, were not considered since the known borinic esters of 8-quinolinol are difficult to hydrolyze.[35] Stabilization by the weaker coordination between boron and oxygen[36] was examined with borinic esters of methoxyethanol, but distillation of the methoxyethanol ester of 2b did not give a pure compound.

The introduction of the coordinating oxygen atom as an ortho-methoxy substituent in the aromatic nucleus of the phenylethyl methyl borinic ester, Compound 6c, was more successful. The interaction between boron and oxygen is apparently strong enough to make purification of borinic ester 6c possible through distillation. The stability of boronic acid 6c in aqueous solution also supports this interaction between boron and oxygen, now in a favorable 6-membered ring structure. A similar interaction between boron and oxygen in the o-methoxybenzylmethyl borinic ester 6b is apparently not strong enough to prevent decomposition of this ester during distillation. Impure fractions were obtained which turned yellow upon standing, as was the case when distilling the parent compound 2b. However, coordination between boron and oxygen in a 5-membered ring structure can occur since the (o-methoxybenzyl) boronic ester 5b is more stable toward autoxidation than the parent compound ester 2b.

Kinetics

All boronic and borinic acids examined (except benzeneboronic acid 1a) are good reversible inhibitors of acetylcholinesterase (see Fig. 1). Results are summarized in Table 1. Inhibition is predominantly competitive, but some compounds appear to show a small noncompetitive component. For comparison, inhibition by acetophenone and α,α,α-trifluoroacetophenone was also examined. Acetophenone is a poor competitive

inhibitor, while trifluoroacetophenone is comparable to most of the boron compounds.

Boronic and borinic esters hydrolyze rapidly and completely in water to the corresponding acid and alcohol.[15] Since n-propanol inhibits the enzyme only at about 10^{-2} M[22], inhibition by lower concentrations of esters may be attributed to the free acids. The agreement between the K_1 values of 33 and 40 μM observed for boronic ester 1c and the reported value of 31 μM for the acid[13] substantiate this conclusion.

Phenylmethylborinic acid (2a) also blocks irreversible inactivation of the enzyme by CH_3SO_2F (Fig. 2). The dissociation constant K_d for compound 2a calculated from this protection experiment is 2.4×10^{-5} M, compared to 2.2×10^{-5} M for competitive inhibition by this borinic acid.

Inhibition of the enzyme by the o-methoxy substituted boronic and borinic acids 5a, 5b, and 6c, required some time to reach a steady state. The effect was not pronounced enough to allow a quantitative kinetic analysis, but half-lives appeared to be of the order of 20 - 30 seconds for both binding to and dissociation from the enzyme under experimental conditions. Inhibition constants given in Table 1 for these compounds were obtained after a steady state had been reached.

Discussion

Synthesis of the boron compounds via literature methods was relatively straightforward. However, susceptibility of the B-C bond to air oxidation limited the range of structures which could be studied as inhibitors. Benzyl methyl and phenylethyl methyl borinic acids could not be obtained pure; phenylisopropyl borinic acid and benzylboronic acid oxidize slowly in water but are sufficiently stable for kinetic studies.

Several kinds of evidence suggest that most of the boron compounds examined are transition state analogs for acetylcholinesterasse. The basic experimental criterion for a transition state analog is that it should bind significantly more tightly than the corresponding substrate.[10] For the compounds 1b, 1c, 5b, 5c, and 6c, comparable substrates for acetylcholinesterase are known. The observed K_m values of 2×10^{-3} M for phenyl acetate and 9×10^{-3} M for benzyl acetate[26] are probably lower limits on the dissociation constants. The K_i value for the benzyl boronic acid 1b is only about 3 times smaller than the K_m of phenyl acetate. The boron compounds 1c, 5b, 5c, and 6c; however, bind 200 - 1500 times more tightly to acetylcholinesterase than the corresponding substrates and thus can be regarded as probable transition state analogs for acetylcholinesterasse. Although no substrate corresponding to the borinic acids 2a and 3a is known, these

compounds do have the same functional boron group and structures similar to those above and are therefore very likely to bind to the enzyme by the same mechanism as the other boron compounds.

Identification of a compound as a transition state analog simply because it binds more tightly than a substrate is not very satisfactory. In earlier work, evidence has been presented that aliphatic ketones are not transition state analogs for acetylcholinesterase, although they bind much better to the enzyme than corresponding substrates.[37] One useful additional criterion in this case is the ability of a compound to block irreversible inactivation of acetylcholinesterase by CH_3SO_2F. This irreversible inhibitor reacts specifically with the nucleophilic serine-OH group. This reaction is not blocked by compounds which bind only in the anionic subsite of acetylcholinesterase.[25]

Blocking of the CH_3SO_2F inactivation of acetylcholinesterase by the borinic acid 2a occurs with the same dissociation constant as K_c for inhibition of enzymatic hydrolysis of acetylcholine. Since borinic acid 2a protects the serine hydroxyl from CH_3SO_2F, the borinic acid probably interacts with or binds near the active site hydroxyl of the enzyme. Because of the structural relationship and related inhibitory properties among the boronic and borinic acids, it is probable that all the compounds examined bind in the same way.

A second useful criterion to determine whether the boron compounds are transition state analogs is comparison to the corresponding alcohols. The closest comparison available is between phenylethyl boronic acid 1c (K_I = 3.7 x 10^{-5} M or the o-methoxyphenylethyl borinic acid 6c (K_I = 1.5 x 10^{-5} M) and 4-phenyl-2-butanol (K_I = 3.6 x 10^{-2} M).[26] The much stronger binding by the boron compounds lends additional support to the interpretation that they bind by nucleophilic addition of the enzyme to boron, rather than by some mechanism involving hydrogen bonding or other interactions of the hydroxyl group.

The occurrence of a small noncompetitive component in inhibition by five of the eight boron compounds suggests more than one mode of binding of these compounds to the enzyme. The active site of acetylcholinesterase is conventionally divided into an anionic subsite which binds the quaternary ammonium group of acetylcholine, an esteratic subsite containing the nucleophilic serine-OH group, and several peripheral subsites which bind various effectors. The mechanism consists of a rapid formation of an acyl-enzyme, followed by its somewhat slower hydrolysis.[11] A noncompetitive component in acetylcholinesterase inhibition by certain quaternary ammonium compounds has been attributed to anionic site binding in the acyl-enzyme[38]; mixed inhibition can also result from binding to peripheral sites.[11,39] Weak binding in either of these sites would not affect our conclusion that these compounds bind predominantly by forming an adduct with the

serine-OH in the esteratic site. However, the possibility remains that the observed competitive inhibition also results from binding in the anionic site, and that binding to this site is weaker in the acyl enzyme for steric reasons. Several considerations suggest that this interpretation is relatively unlikely. First, effective protection of the esteratic serine-OH from reaction with CH_3SO_2F by borinic acid 2a is hard to reconcile with binding of the borinic acid only in the anionic site. Most compounds which unambiguously bind at the anionic site either have little effect on CH_3SO_2F inactivation (e.g., phenyltrimethylammonium ion) or accelerate inactivation (tetramethylammonium ion).[25] Although the poor substrate p-nitrophenyl acetate[40] and the bulky tetrabutylammonium ion[25] provide some degree of protection against CH_3SO_2F, the protection is relatively inefficient - concentrations higher than the binding constant are required to protect the serine-OH. The only clear example of a compound which binds at the anionic site but efficiently protects against CH_3SO_2F is 3-hydroxyphenyltrimethylammonium ion[25] - other evidence suggests that the hydroxyl group of this inhibitor interacts with the esteratic site. Second, the compounds examined differ considerably in steric bulk - if they bound only in the anionic site, some instances of predominantly noncompetitive inhibition might be expected. Third, the borinic acid analog of acetylcholine is a very potent inhibitor[13] - its structure and pH dependence for binding imply formation of an adduct between

boron and the serine-OH rather than the anionic site. Fourth, uncharged aliphatic ketones which inhibit but are not transition state analogs protect the enzyme very poorly from CH_3SO_2F.[37] While binding of the boron compounds only to the anionic site cannot be ruled out by these arguments, it seems a relatively implausible interpretation.

Some empirical structure - activity correlations are also apparent. Increasing chain length in phenylalkylboronic acids leads to increasing inhibition, from benzeneboronic acid 1a with K_i > 1 mM to phenylethylboronic acid 1c with K_c = 37 μM. Similarly, alkyl substitution on boron leads to increasing inhibition from 1a to phenylmethylborinic acid 2a (K_c = 22 μM) to phenylisopropylborinic acid 3a (K_c = 4.4 μM). Qualitatively, all of these effects can be rationalized by assuming a correlation between increased hydrophobicity and increased inhibition. Quantitatively, specific binding effects may also be important, and a difference in electrophilicity between boronic and borinic acids may account for the enhanced binding of 2a compared to 1a.

Compared to the unsubstituted analogs, the o-methoxy compounds 5b, 5c, and 6c are more stable chemically, are better acetylcholinesterase inhibitors, and show time-dependent inhibition. Hydrolysis experiments with boronic esters 5b and 5c and the borinic ester 6c showed that they hydrolyze as readily in water as do the corresponding unsubstituted compounds. Thus, the active species in aqueous solutions of these esters

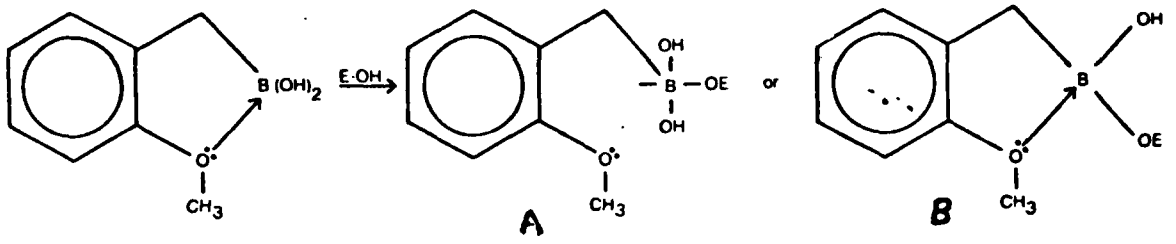
are the free acids. For the borinic acid 6c, this was also demonstrated by the color reaction with diphenylcarbazone.

Coordinative B←O interaction between the methoxy group and boron in the free acids is the most likely explanation for the greater stability of the boronic acid 5b and the borinic acid 6c toward autoxidation in dilute aqueous solution, when compared to the unsubstituted boronic acid 1b and the very air-sensitive borinic acid 2c.

Internal B←O coordination also probably accounts for the time-dependent inhibition of acetylcholine hydroylsis seen with the o-methoxy compounds 5b, 5c, and 6c.

The slow binding and dissociation of these inhibitors (half-lives of 20 - 30 seconds for both processes) provides additional evidence for formation of a covalent complex with the enzyme. The complex expected by analogy with the unsubstituted boron acids would be the tetrahedral adduct (A in Scheme II), but a formally neutral borinic or boronic ester such as B in Scheme II is an attractive alternative for the o-methoxy compounds. The slow dissociation observed is difficult to rationalize in terms of the tetrahedral adduct A which might be expected to dissociate at the same rate as adducts of unsubstituted boronic acids. Slow formation and hydrolysis of ester complex B could account for the observed time-dependence of inhibition. Although boron esters

Scheme II



ordinarily react rapidly with water, their hydrolysis rate is very sensitive to steric hindrance; for example, borate esters of large, tertiary alcohols hydrolyze with half-lives of the order of 1 - 100 hours.[41] Thus, it would not be surprising if ester complexes such as B hydrolyze with a half-life of the order of 20 - 30 seconds in the hindered environment of an enzyme active site. Less quantitative data is available on the rate of esterification of boron acids but similar considerations would probably apply. Formation of an ester complex such as B could then explain both slow binding and dissociation rates.

The observation that the o-methoxy boronic acids are better inhibitors than their unsubstituted analogs is also consistent with formation of an enzyme-inhibitor complex such as B in which methoxy remains coordinated to boron. If B$\leftarrow$O coordination contributes extra stability in solution but not in the adduct with the enzyme (Structure A), then the o-methoxy compounds should not inhibit as well as their unsubstituted analogs.

Other effects of o-methoxy substitution appear in specificity of these inhibitors and may reflect several factors. For instance, phenylethylboronic acid 1c is a much better inhibitor than benzylboronic acid 1b, but the o-methoxy compounds 5b and 5c are almost equally potent. This difference in specificity may result partly from a hydrophobic or other attractive interaction between the o-methoxy group of 5b and the enzyme. Such an interaction occurs in the geometrically similar phenyl N-methyl carbamates, where an o-methoxy substituent enhances binding to bovine erythrocyte actylcholinesterase by a factor of 7.[42] A second factor probably influences binding of borinic acid 6c; B O coordination in this molecule makes the boron atom chiral. If only one of the two enantiomers inhibits the enzyme, the dissociation constants for borinic acid 6c and boronic acid 5c become almost equal.

Conclusions

Most of the boronic and borinic acids investigated are potent acetylcholinesterase inhibitors and appear to be transition state analogs. They are among the most powerful competitive inhibitors of this enzyme except for a few quaternary ammonium compounds which interact specifically with the anionic site. The most nearly comparable class of compounds are structurally analogous electrophilic ketones, which inhibit with similar dissociation constants.[14] For example,

phenylmethylborinic acid and trifluoroacetophenone are equivalent as inhibitors. It is likely that introduction of substituents such as trimethylammonio, isopropyl, isopropoxy, or others known to interact with this site, into either class of compounds, should lead to extremely powerful inhibitors of possible pharmacological interest.

If the rough equivalence of boron acids and electrophilic ketones as acetylcholinesterase inhibitors also proves to be true for other esterases and proteases, then the choice of a functional group for a transition state analog will be dictated by synthetic considerations. The present work, along with the pioneering synthetic work of Torssel and others,[15] suggests some guidelines. Electrophilic ketones, aldehydes, and borinic acids should all be suitable for analogs of the type R-E, where R is a group conferring specificity, and E is an electrophilic group. Ketones and aldehydes may prove more useful in some cases because there are more methods available for their synthesis. For analogs of the type R-E-R, either electrophilic ketones or borinic acids fit the structural criteria. The present results emphasize the limited stability and susceptibility to oxidation of borinic acids, extending some reports in the earlier literature. However, this work also indicates that coordination with a weak electron donor such as the o-methoxy substituent can overcome this stability problem in some cases, as well as substantially increase the inhibition observed.

Table I

Reversible Inhibition of Acetylcholinesterase

The dissociation constant K_c for competitive inhibition and the ratio of constants K_c/K_n for competitive and noncompetitive inhibition were measured at pH 7.5, 25°, in solutions containing 0.1 M NaCl and 0.04 M $MgCl_2$.

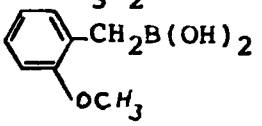
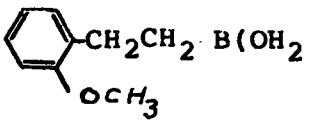
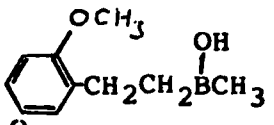
Structure	Number	K_c (μM)	K_c/K_n
$C_6H_5B(OH)_2$	1a	> 1000	---
$C_6H_5CH_2B(OH)_2$	1b	800	---
$C_6H_5CH_2CH_2B(OH)_2$	1c	37	---
$C_6H_5B(OH)CH_3$	2a	22	0.07
$C_6H_5B(OH)CH(CH_3)_2$	3a	4.4	0.11
	5b	4.0	0.04
	5c	5.8	0.08
	6c	15	0.09
$C_6H_5CCH_3$ (O)		1500	---
$C_6H_5CCF_3$ (O)		20	---

Figure 1.

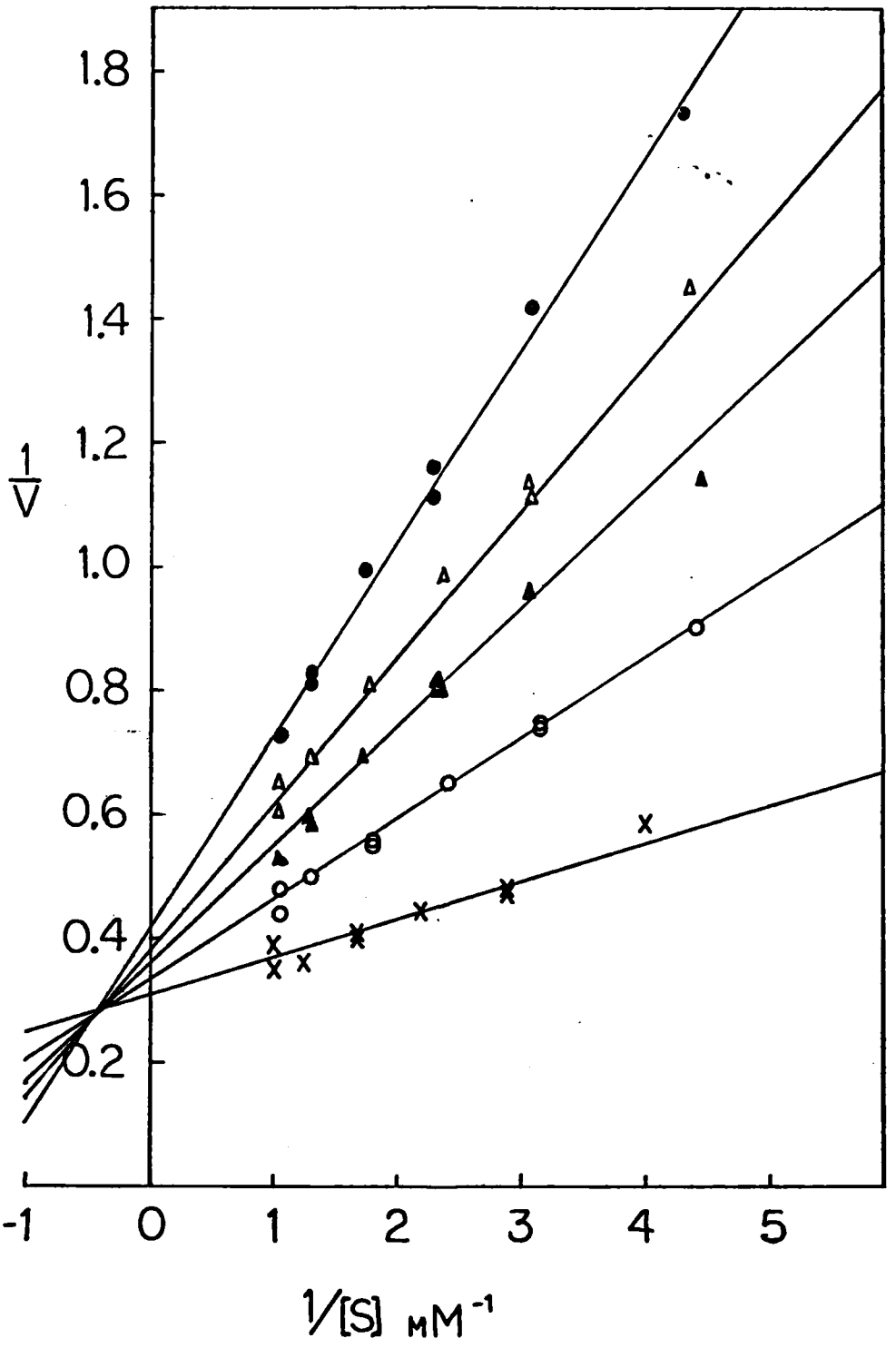


Figure 2

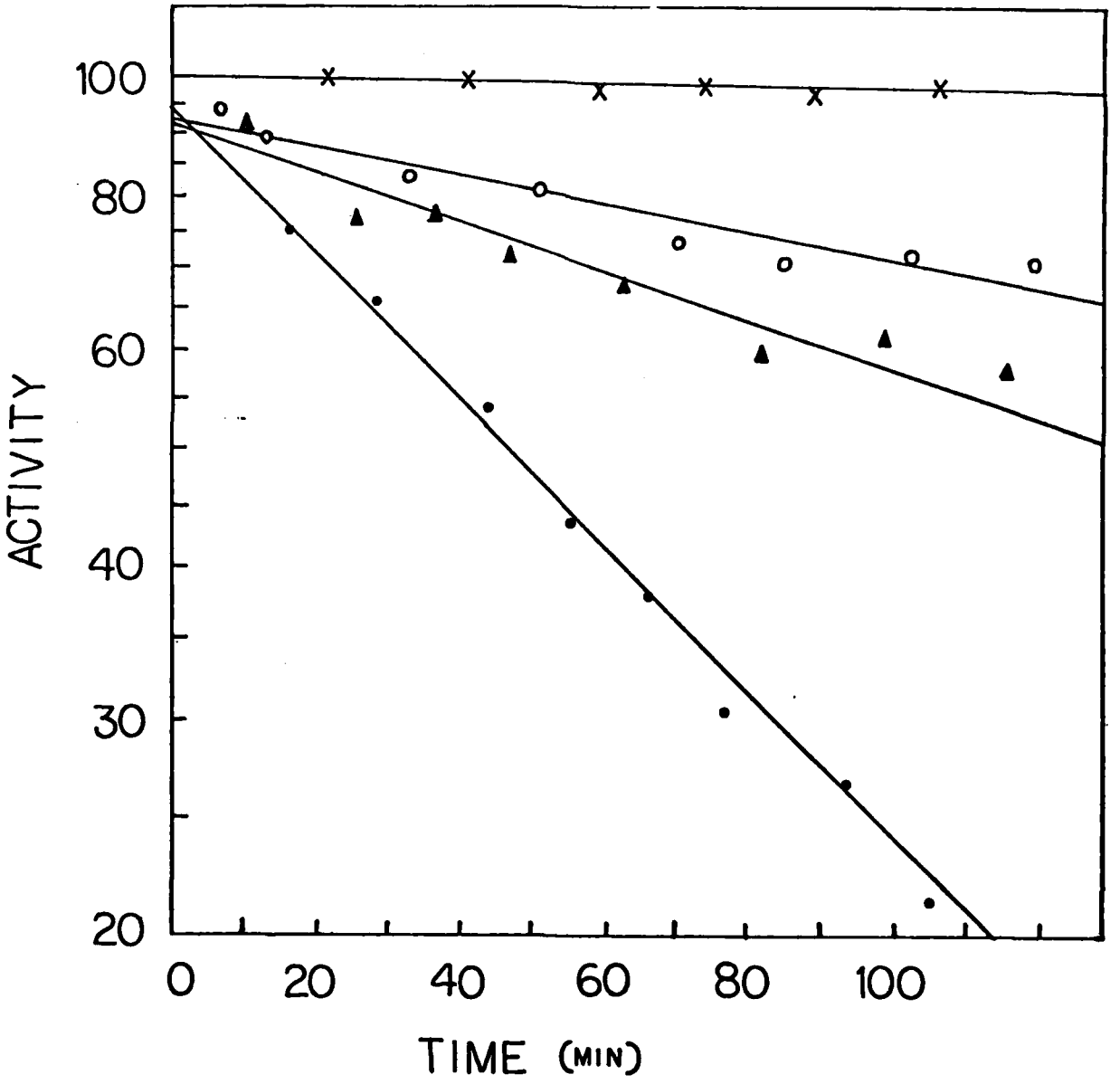


Figure Legends

Figure 1. Inverse velocity vs. inverse substrate concentration without inhibitor (X) and in the presence of 15 uM (0), 30 uM (▲), 45 uM (Δ), and 60 uM (●) compound 6c.

Figure 2. Activity vs. time without methanesulfonyl fluoride (X), with 0.1 mM methanesulfonyl fluoride (●), and with 0.1 mM methanesulfonyl fluoride plus 0.049 mM (▲) or 0.095 mM (O) compound 2a.

APPENDIX III

7-Trifluoromethanesulfonoxyquinoline (6). To a stirred chilled solution of 1.0 g (6.9 mmole) of 7-quinolinol (Eastman) and 0.83 ml (7.1 mmole) of 2,6-lutidine (Sigma) in 7.0 ml of $CHCl_3$ was added 1 ml (9.4 mmole) of trifluoromethanesulfonyl chloride (Aldrich) in two 0.5 ml portions over 20 min. The reaction mixture was allowed to come to room temperature and stirring was continued overnight. The mixture was refluxed for 20 min then the solvents were removed by rotary evaporation. After vacuum desiccation the crude product, a tan crystalline powder, weighed 1.35 g. Recrystallized from ligroin containing a little EtOAc, after charcoal treatment to give 1.0 g of yellow-white crystals, mp 75-76°. Recrystallized again from ligroin-EtOAc to yield, after vacuum desiccation, 0.83 g (43%) of whit ecrystals, mp 75-76°. R_f = 0.68 on silica gel tlc in $CHCl_3$-MeOH (8:1,v/v). IR spectrum compatible with structure (see text). NMR ($CDCl_3$, TMS) 7.8 δ (m, 5H), 8.9 δ (pair of doublets, 1H, proton at C-2).

1-Methyl-7-trifluoromethanesulfonoxyquinolinium Iodide (2). A stirred solution of 0.5 g (1.8 mmole) of compound 6 in 4 ml of acetone was treated with a total of 0.62 ml in three 0.14 ml aliquots and one 0.2 ml aliquot over three days. Except for a one-hour reflux on the second day, the mixture was kept at room temperature. Addition of ether gave a yellow precipitate, which when dried under vacuum weighed 0.27 g (36%), mp 148-151° dec, A 100 mg sample was recrystallized from a mixture of acetone, EtOAc, and Et_2O (approx. 4 ml, 4 ml, and 3 ml respectively) to give 70 mg of yellow powder mp 153-154, dec. Both the crude product and the recrystallized sample were homogeneous on 250 micron Avicel F (Analtech) developed in 1-BuOH:EtOH:H_2O (10:3:7, v/v). R_f = .074. Mass spectrum showed M-CH_3I peak at m/z = 2.77. UV λ_{max} pH 7.4 = 314 nm (log ϵ = 3.91). Analysis: calculated for $C_{11}H_9F_3INO_3S$ (MW = 419.18): C, 31.5%; H, 2.16%, F, 13.60%. Found: C, 31.34%; H, 2.22%; F, 13.48%.

3-Trifluoromethanesulfonoxy-N,N-dimethylaniline (8). Recrystallized 3-dimethylaminophenol (Aldrich) from ligroin after charcoal treatment to give off-white crystals, mp. 85-87°. To a stirred, chilled (ice bath), solution of 2.37 g (17.25 mmole) of the phenol and 3 ml of 2,6-lutidine in 12 ml $CHCl_3$ was added dropwise over 0.5 hr a solution of 4.35 ml (25.9 mmole) of trifluoromethanesulfonic anhydride (Aldrich) in 4 ml $CHCl_3$. The mixture was then refluxed for 30 min. Triethylamine, 3.6 ml (25.9 mmole), was added. Extracted with 20 ml of 10% Na_2CO_3, then with 20 ml of H_2O. The dark purple organic layer was dried over $MgSO_4$. Rotary evaporation removed $CHCl_3$ some lutidine. The remaining lutidine was removed under high vacuum. Distillation gave 2.2 g of liquid, boiling range 83-110° at 0.05 mm Hg. The product had some purple color, but was homogeneous by tlc on silica gel in EtOAc and had a compatible ir (neat) spectrum (see Text). Mass spectrum: M^+ at m/z = 269, CF_3 fragment peak at m/z = 69.

(3-hydroxyphenyl)trimethylammonium iodide trifluoromethanesulfonate (4). To a stirred solution of 2.0 g (7.4 mmole) of compound 8 in 4 ml of acetone was added 1.16 ml of CH_3I. After 20 min the mixture was refluxed for 15 min. The reaction was monitored by tlc on silica gel in EtOAc. More CH_3I (2.0 ml) was added. Refluxed again for 15 min during which time a yellow precipitate formed. CH_3I (2.0 ml) was added, refluxed 30 min, and refrigerated overnight. Ligroin (approx. 6 ml) was added to induce more precipitate to form. The product was collected by filtration under suction and dried to yield 0.5 g of gray-white crystals, mp. 154-157°(dec) solvents were removed from the filtrate by rotary evaporation, and the residual yellow oil (1.56 g) was dissolved in neat CH_3I (8 ml). After refluxing for 2.5 hr, stirring was continued for 2 days at room temperature. CH_3I was removed by rotary evaporation. The residue was tritrated with ether and the pale yellow product was collected by filtration under suction and dried in a vacuum desiccator to give 0.43 g of pale yellow powerd, mp.

The nmr spectrum gave a pattern consistent with the structure of the compound (see next page); multiplet 9.7-8.18 δ (4H), singlet 4.4 δ (3H). The extra peak is water impurity. The HPLC chromatogram of the compound in methanol was a single sharp peak with a retention time of five minutes on an Altex C-18 reverse phase, 4.6 mm ID column (5μ particle size). Elemental analysis: Calculated: %C: 22.779, %H: 1.1912, %F: 15.422, %N: 3.795, %O: 13.004, %S: 8.686, %I: 34.382. Found: %C: 22.88, %H: 1.94, %F: 15.16. The small discrepancy (1.6%) in the Fluorine analysis was attributed to a minor degree of hydrolysis giving 3-hydroxy-N-methyl pyridinium iodide. The nmr spectrum of the product and the HPLC chromatogram are reproduced on the next two pages.

149-153°. The combined product (0.93 g) was recrystallized from approx. 10 ml MeOH after charcoal treatment to give 70 mg of white crystals, mp 156.5-157.5° (dec), from which a sample was submitted for elemental analysis. A second crop of large crystals fro MeOH weighed 486 mg with mp. 156.5-158.5° (dec). Both crops were homoegenous by tlc on 250 micron Avicel F in 1-BuOH:EtOH:H_2O (10:3:7, v/v). R_f = 0.76. Mass spectrum: M-CH_3I peak at m/z = 269. UV $_{max}H_2O$ = 225 nm (log = 4.10), shoulder at 260 nm (log ϵ = 3.06). Analysis: calculated for $C_{10}H_{13}F_3INO_3S$ (MW = 411.18): C, 29.21%; H, 3.19%; N, 3.41%. Found: C, 29.24; H, 3.25%; N, 3.36%.

3-trifluoromethanesulfonyloxy-N-methyl pyridinium iodide.

The procedure is that used by Ginsburg (56) for the preparation of 3-methanesulfonyloxy-N-methyl pyridinium iodide.

.06 mol 3-hydroxypyridine were placed in a 50 ml round bottom flask in 6.0 ml chloroform (distilled and dried). 2.6 lutidine (.72 ml) was added and a dropping funnel was placed over the solution. A slight excess of trifluoromethanesulfonyl chloride (10.0 ml) in chloroform was added to the funnel; then added slowly to the pyridine compound. The mixture was cooled while stirring to keep the trifyl chloride from boiling away. After stirring for about twenty minutes, the mixture was allowed to sit overnight. The next day, the yellow solution was refluxed for twenty minutes, then extracted 3 times with water to remove the lutidine hydrochloride. The chloroform layer was dried over sodium sulfate and then the solvent distilled off. The dark orange residue was fractionated through a short Vigreux column equipped with a condenser under vacuum (.5 Torr). The first fraction came over at 23°C, and was a clear liquid whose nmr indicated the correct product. Yield was 52%.

3-trifluoromethanesulfonyloxy pyridine was quaternized in acetone at room temperature by adding excess methyl iodide from a dropping funnel with stirring. A yellow color quickly developed. After addition of methyl iodide was completed, the mixture was refluxed briefly (10.0 min.) and allowed to stand overnight. No crystals were evident until the next day; agitation caused a yellow precipitate to fall out of the orange-colored solution. The melting point of the crude product was 135-139°C. It was recrystallized from ether-acetone; light fluffy yellow crystals were obtained and dried under vacuum. Yield was 78% and the melting point was 140-145°C (decomp.). No literature M.P. was available as there is no previous preparation.

Appendix 4

ACYLSILANES AS POTENTIAL PHOTOAFFINITY LABELS: ACTIVE-SITE-DIRECTED PHOTOINACTIVATION OF ACETYLCHOLINESTERASE BY 1-NAPHTHOYLTRIMETHYLSILANE.

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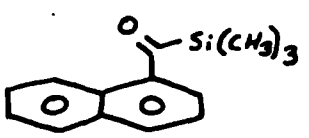
Photoaffinity labelling has become a widely used biochemical technique in the last decade (1). Most photoaffinity labels work by generating reactive carbenes or nitrenes, which rapidly form covalent bonds within binding regions of a protein or other biological target molecule. However, such systems often have serious limitations. One problem is internal rearrangement of the carbene or nitrene to a less reactive species, for example the rearrangement of α-ketocarbenes to ketenes. Such a rearrangement makes reaction with and labelling of the biological target molecule much less likely. A second drawback is that many of the carbene and nitrene precursors must be photolyzed at wavelengths as short as 250-280 nm. Most proteins and some other biomolecules absorb light at these wavelengths, limiting the effectiveness of the experiment and possibly resulting in structural damage to the target molecule.

Photoaffinity labels based on acylsilanes (α-silyl ketones) could overcome these problems since on photolysis at wavelengths as long as 400 nm acylsilanes normally generate siloxycarbenes which can be efficiently trapped in bimolecular reactions (2). In the absence of trapping reagents, the siloxycarbene reverts back to the starting acylsilane allowing carbene formation to occur again. We now wish to report that 366 nm irradiation of a mixture of the acylsilane α-naphthyl trimethylsilyl ketone (1-naphthoyltrimethylsilane), 1, and acetylcholinesterase (AChE) results in rapid deactivation of the enzyme.

This acylsilane was chosen as a candidate photoaffinity label for AChE because of the known affinity of the enzyme for α-naphthyl substrates and inhibitors, particularly the insecticide Carbaryl (the N,N-dimethylcarbamate of α-naphthol). α-Naphthyl trimethylsilyl ketone (1) was prepared and its purity demonstrated by standard methods. Irradiation of 1 in methanol yields acetal 2, clearly consistent with rearrangement of an excited state of 1 to siloxycarbene 3 (2).



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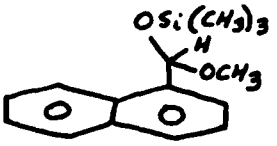

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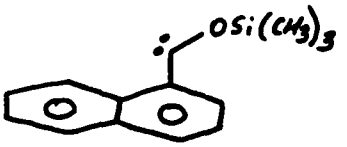

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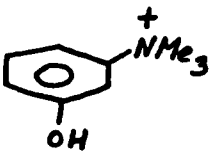

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Inactivation experiments were conducted in the following manner. Stock solutions of the acylsilane in methanol were used to prepare 1% methanol (v/v) solutions containing various concentrations of acylsilane, 0.1M NaCl, 0.04M $MgCl_2$, 1mM phosphate buffer (pH 7.5), and 1-2 units/ml of Electric Eel AChE (Sigma Chemical Co., > 1000 units/ml). Aliquots (5 ml) of this solution were placed in 10 x 75 mm Pyrex test tubes and irradiated in a carousel reactor using a 450W medium pressure mercury lamp equipped with filters which isolate the 366 nm region (3). Aliquots (1 ml) of the reaction mixture were withdrawn at various times and assayed for remaining enzyme activity by diluting to 30 ml with a solution containing 1.0 mM acetylcholine, 0.1M NaCl, 1 mM phosphate buffer, pH 7.5, and 0.04M $MgCl_2$. The rate of acid production was then monitored using a pH Stat. Details of this assay have been published elsewhere (4).

For most experiments, the initial concentration of acylsilane was 1.25×10^{-4} M; at this concentration appreciable turbidity was apparent. Typically 80 to 90% inactivation was observed after 5 minutes of irradiation. A small loss of activity in the control which contains no acylsilane probably results from heating, since AChE is reported to be stable to photoinactivation at wavelengths greater than about 310 nm. Other controls established that the inactivation was a direct consequence of the photolysis of acylsilane and the enzyme. For example, incubation of the enzyme with acylsilane in the absence of light does not lead to inactivation. Further, if the acylsilane is first photolyzed to completion (6-7 minutes, monitored by following the UV-Visible absorption at 380 nm) and enzyme is then added, no inactivation is observed even after continued photolysis. Thus the observed inactivation is not due to photolytic or nonphotolytic inactivation by stable acylsilane photolysis products.

The observed inactivation appears to be active-site directed, since photolytic inactivation is much less efficient in the presence of the good competitive inhibitor m-N,N,N-trimethylammoniophenol, 4. For example, irradiation under the same conditions noted above except that 1.5×10^{-6} M m-N,N,N-trimethylammoniophenol had been added to the photolysis solution, decreased the observed photoinactivation from 92% to 30%. The acylsilane itself, when included in a normal assay mixture at a concentration of 1.25×10^{-4} M, causes barely detectible competitive inhibition.

The photoinactivation is specific for the α-naphthyl structure, since the β-naphthyl isomer (2-Naphthoyltrimethylsilane) is much less effective and acetyldimethylphenylsilane is completely ineffective as a photoinactivator of AChE. Control experiments have indicated that photoinactivation of AChE by 1 also occurs in the absence of oxygen, ruling out photo-oxidation of the enzyme as the cause of the observed inactivation.

Photoinactivation is also observed at higher concentrations of 1, up to 5×10^{-4} M, but in this case the kinetics appear to be nonlinear, with about 50% inactivation occurring in the first minute, followed by a slower rate of inactivation. This rapid initial process has not yet been investigated.

The most plausible interpretation of our data is that photolysis

of the silane results in formation of a siloxycarbene, which inserts into a bond in the enzyme active site resulting in inactivation of the enzyme. Experiments are currently in progress to determine whether the acylsilane or the siloxycarbene derived therefrom is actually incorporated stoichiometrically into the enzyme.

In summary, α-naphthyl trimethylsilyl ketone appears to be a specific photoinactivator and possibly a photoaffinity label for AChE. The inactivation is rapid and potent, suggesting that appropriately designed acylsilanes may be suitable as photoaffinity labels for other biological systems. As noted earlier, the main advantages of acylsilanes over other classes of compounds are their long wavelength absorption, fairly rapid photolysis, and the fact that the intermediate carbene rearranges only to re-form the starting material. Potential disadvantages include the relatively large size of the trimethylsilylacetyl group, which might cause steric problems with binding, the limited number of methods which are available for synthesis of these compounds, especially in the presence of other functional groups, and the fact that the siloxycarbene intermediates generated photochemically from acylsilanes are more stable and less reactive than many other carbenes. For example, they do not add to carbon-carbon double bonds or under usual conditions, insert into carbon-hydrogen single bonds, but instead show a marked preference for insertion into acidic heteroatom - hydrogen bonds (2). Nevertheless, this affords considerable scope for insertion into protein backbones or amino acid side chains, making the properties of acylsilanes as photoaffinity labels appear well worth exploring.

REFERENCES

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2. See, for example, (a) A. G. Brook, Intra-Sci. Chem Rep., 7, 131 (1973); (b) R. A. Bourque, P. D. Davis and J. C. Dalton, J. Am. Chem. Soc., 103, 697 (1981); (c) J. C. Dalton and R. A. Bourque, J. Am. Chem. Soc., 103, 699 (1981) and references therein.

3. A combination of Corning Glass Works 0-52 and 7-60 filters was used.

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